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## PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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60/423368

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<input checked="" type="checkbox"/> Additional inventors are being named on the <u>1</u> separately numbered sheets attached hereto		
TITLE OF THE INVENTION (500 characters max)		
ULTRASONIC LOW ENERGY TREATMENT TRIGGERING APOPTOSIS OF HUMAN CANCER CELLS		
Direct all correspondence to:		
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**PROVISIONAL APPLICATION COVER SHEET**  
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### Induction of apoptosis in human leukemic cells by ultrasonic low energy treatment.

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The biophysical effects of ultrasound have been frequently surveyed and its use has been proposed for some years, often in conjunction with other energy types (hyperthermia, radiation) for the treatment of cancer cells. This procedure requires long preparation, energy and the use of photosensitizers. In contrast, the technique described here, using diagnostic ultrasonic frequencies, requires a low acoustic power level and, in the absence of any chemical synergy, triggers apoptosis in leukemic cells. 2 hours after ultrasonic treatment of K562 cells, a sequence of events recognized as hallmarks of apoptosis was observed: depletion of glutathione, a drop of mitochondrial potential, translocation of phosphatidylserine and, subsequently, loss of membrane integrity. These effects are not dependent on the formation of species issued from the sonolysis of the solvent medium. By using minute amounts of energy, necrosis generally associated with a high physiological stress is minimized (<10%). With successive irradiations, under the same conditions (7mW/ml, 20 sec) and at different intervals, K562 cell apoptosis was significantly increased (more than 25% after 2 irradiations). The higher sensitivity of K562 cells compared to normal blood cells allows a therapeutic index dose to be determined. Moreover, the sensitivity of ultrasound seems to depend on the cell type. K562 cells and B-CLL cells are the most sensitive compared to HL-60 cells and normal mononuclear blood cells. Thus, in our conditions, the cell damage induced by ultrasound in leukemic cells occurs through apoptosis and offers a mechanism of the smooth elimination of leukemia cells.

**ULTRASONIC LOW ENERGY TREATMENT TRIGGERING APOPTOSIS  
OF HUMAN CANCER CELLS**

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**ABSTRACT**

The biophysical effects of ultrasound have been frequently surveyed and its use has been proposed for some years, often in conjunction with other energy types (hyperthermia, radiations) for the treatment of cancer cells. The preparation time is long, heavy energy use is necessary and normally, photosensitisers need to be administered. The technique discussed here, using diagnostic ultrasonic frequencies, requires a low acoustic power level and, in the absence of any chemical synergy, triggers apoptosis specifically for the selective elimination of cancers cells. By using minute amounts of energy, necrosis, generally associated with a high physiological stress, is minimised. Indeed, this treatment leads to a sequence of events recognised as hallmarks of apoptosis: a drop in mitochondrial potential, translocation of phosphatidylserine of the plasma membrane and subsequently loss of membrane integrity. The effects obtained within a very short time implicate the topical photosensitisers of the cells and emphasise the major role played by singlet oxygen. The higher sensitivity of cancer cells, compared to normal blood cells, allows therapeutical application. The sensitivity of normal peripheral blood and malignant cells (K562, HL60, B-CLL) was compared.

## INTRODUCTION

The use of ultrasound irradiation in cancer therapy has become an important issue (1-6). Ultrasound have been used in conjunction with hyperthermia, or photo-, radio- and chemo-therapy (7,8). Malignant cells are known to be more susceptible to these combined methods than their normal counterparts. Recently, intrinsic effects of ultrasound have been demonstrated for weak powers and irradiation time of few minutes (2). The effect of a direct irradiation (ultrasound, micro-waves, laser, light etc...) on certain molecules (like porphyrines, porphynes, porphycenes, phthalocyanines, levulinic acid derivatives, psoralenes, anthracyclines...) is to generate highly active oxygen species such as, among others, singlet oxygen, superoxyde radical or fatty acid radicals (9) which can play an important role in cancer treatment, acting selectively on malignant cells (3).

In the case of a luminous irradiation, these molecules, administered either by injection or orally, form oxidising species as a result of the photosensitization from the cellular oxygen. According to the origin of the photons, this is called PDT (photodynamic therapy) or, if by sonoluminescence (sometimes called the luminescence acoustochemical effect), called SDT (sonodynamic therapy). The photopheresis requires high luminous intensities. This is not the case for sonoluminescence.

Singlet oxygen plays a major role in pathological disorders and in the apoptotic process (10), its relevance lies in both its activity ant its ability to be produced in these systems. The power of singlet oxygen ( ${}^1\text{O}_2$ ) in biological systems is primarily due to its high ability to be formed by photosensitization. In condensed systems such as the lipidic matrices, peroxidation of the fatty polyunsaturated acids (PUFA) are initiated by the singlet oxygen and generate a cascade of oxidative reactions. Once

peroxidation has been initiated by the singlet oxygen it becomes rapidly autocatalytic in the presence of the oxygen in its fundamental triplet state (11).

Photosensitised oxygenation is characterised by : a non-generation of free radicals; no induction period and a very small energy involved (this resulting in a modification of the cis-trans configuration of the double C-C link of the unsaturated lipids). The lipidoperoxidation, resulting from the modification of the phospholipid membranes plays an essential role in the cell biology and in the induction of apoptosis (12,13). In fact, many of the inducing agents of apoptosis are either oxidants or stimulants of the oxidative cell metabolism. Compared to the generally observed necrotic effects of high energy processes, one of the major characteristics of apoptosis seems that it is always initiated by a low energy solicitation, direct or indirect, physiological or external effects, (PDT, UV, laser,...)(4,14-17). Unlike necrosis, apoptosis does not create either oxidative stress or inflammation.

The effects generated by SDT and PDT are different in terms of surviving cell viability (4); both SDT (specifically related to the ultrasonic cavitational activity) and PDT generate active oxygenated species and lead in each case to a diminution of the intracellular thiol levels. In the case of PDT by UVA, apoptosis of T Helper cells is induced by the generation of singlet oxygen. *In vitro*, this apoptosis seems to be mediated through the FAS/FAS ligand system (18). However, the apoptotic effect is not directly proportional to the light intensity but depends on the initial concentration in photosensitisers (PS) and on the local oxygen concentration (19). For SDT, as a result of the high energies involved, the cell lysis is the major phenomenon, probably masking other effects on the surviving cells (20). The ultrasound initiates a physiological stimulus that involves active oxygenated species in inducing apoptosis in the same way that ionising radiations are capable of inducing oxidative stress (21).

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Classical SDT leading to apoptosis involves specific sensitising molecules, requires an electrical power of about  $5\text{W/cm}^2$  and irradiation time of several minutes, whereas in the process described in this present work, the effects are obtained in a few seconds with powers of about  $0.2\text{ W/cm}^2$ . Since in our case it is sonoluminescence that triggers the production of singlet oxygen that in turn can explain the various secondary effects noted in the specific induction of apoptosis, we have named our technique SLDT : Sonoluminescence Dynamic Therapy.

## MATERIALS AND METHODS

### Cell preparation:

The K562 human chronic myelogenous leukemia cells and the HL-60 promyelocytic leukemia cells obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) were grown in RPMI-1640 (Bio Wittacker, Maryland, USA) supplemented with 10% fetal calf serum (Gibco, New-York, USA) and 1% L-glutamine (Gibco). Cells were subcultured twice a week and kept in exponential growth. K562 or HL-60 cells were harvested, re-suspended in phosphate buffered saline (PBS pH=7.2, Gibco) and immediately used for the experiment at a concentration of  $10^6$  total. Heparinized venous blood was obtained from healthy volunteers and B-cell chronic lymphocytic leukemic patients after informed consent was obtained. Mononuclear cells were separated by Ficoll-Hypaque gradient density centrifugation (International Medical Products, Brussels, Belgium).

### Ultrasonic treatment:

The ultrasonic treatment and its specific results are part of the Patent PCT/BE97/00078 (pending) under the name of Eric Cordemans de Meulenaer & al. A total of 2.5 ml of cell suspension in 13x100-mm disposable plastic tubes (Greiner, Labortechnik, Wemmel, Belgium) was treated in our system with a frequency of 1,8 MHz during various times of exposure. Unless indicated otherwise, the power supplied by the generator to the ceramic disk is 0.22 W/cm<sup>2</sup> – which represents an acoustical power transmitted to the interior of the test cell of 0.007 W/ml. This calorimetric measurement was made at the University of Coventry -England- under Professor Tim Mason. The irradiation (ON/OFF) cycles are of 5.5 ms/3 ms. As shown in figure 1, the ultrasonic source is placed at the bottom of a reactor filled with

degassed water. The tube containing the cells in suspension is placed in the water in such a way as to ensure that the levels are the same in the cell and the tube. A capillary tube placed at the bottom of the cell provides a continuous stream of microbubbles during the irradiation. The distance between the transmitter and the bottom of the tube is 3 cm. The ultrasonic transmissions are axial and can be verified by the acoustic fountain formed in the test tube. Cells were also sonicated, for the experiments undertaken at a frequency of 35 kHz, with the same tube assembly in the standing wave field at the centre of a sonication bath (Bandelin, Berlin, Germany).

Cell viability:

The cell viability was assessed by the trypan blue exclusion test at once after ultrasonic treatment and after 18h culture in the incubator (37°C and 5% CO<sub>2</sub>). In some experiments, the dead cell count was also estimated by flow cytometry using the propidium iodide (PI) assay.

Morphological studies:

To assess morphologic changes after ultrasonic treatment, cytocentrifuge preparations were made from the cell suspension. After air drying, cells were stained with May Grinwald Giemsa and analysed by light microscopy.

Annexin V binding assay:

Flow cytometric analysis of annexin-V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) stained cells was performed using the kit purchased from Biosource International (Camarillo, CA) as recommended by the manufacturer. Briefly, cells were washed with PBS and re-suspended in 1X binding buffer. 5 µl of FITC-Annexin V and 10 µl of PI were added to the cells for 15 min at room temperature. After incubation, 400 µl of binding buffer were added and cells were

analysed immediately. Data are presented as dot plots showing the change in mean fluorescence intensity of annexin-V-FITC/propidium iodide.

DNA fragmentation:

Quantification of cells with degraded DNA was performed using a method described by Nicoletti et al (22). Cells to be analysed were collected, washed, permeabilised, and incubated with solution containing propidium iodide (PI) and RNase (Coulter DNA-prep Reagent). The tubes were placed at 4°C in the dark overnight before analysis by flow cytometry to identify the sub-G0 peak corresponding to apoptosis.

Mitochondrial damage:

Mitochondrial potential was estimated by incorporation of the cationic fluorochrome DIOC6 immediately after cell treatment according to a published protocol (23). Briefly, K562 cells ( $10^6$ /ml) were incubated with 2.5 nmol/L 3,3'-dihexyloxacarbocyanine (DIOC6 ; Molecular Probes, Eugen, OR) for 15 min at 37°C followed by flow cytometric analysis.

Glutathione determination:

Cell Tracker green CMFDA (5- chloromethyl fluorescein diacetate) (Molecular Probes) was used for determining levels of intracellular glutathione as previously described (24). Glutathione is rapidly lost once the surface membrane is compromised and for this reason, this assay requires intact cells. Glutathione level was thus evaluated in viable cells (PI negative cells).

## RESULTS

We observe that the ultrasonic treatment induces a series of successive phases, classical and specific to the induction of apoptosis. 2 hours after ultrasonic treatment: depletion of glutathione already observable; 5 hours after ultrasonic treatment: perturbation of the mitochondrial potential, loss of phosphatidylserine asymmetry; 5h to 18h after ultrasonic treatment : appearance of PI<sup>+</sup> cells.

It should be noted that no induction effect was observed when the ultrasonically pre-treated medium was subsequently added to the cells demonstrating that the effects described above are not dependent on the formation of long living species issued from the sonolysis of the solvent.

### Loss of phosphatidylserine asymmetry during ultrasonic treatment:

During apoptosis, phosphatidylserine residues flip from the inside to the outside of the plasma membrane and this change can be detected using annexin-FITC which binds to the PS residues (25). The addition of PI allows the evaluation of the membrane permeabilisation and the later development of apoptotic cells. Figure 2 indicates the change of phosphatidylserine distribution according to time. K562 cells were treated during 20 sec, washed and stained by annexin/PI directly or after several periods after the ultrasonic treatment. Ultrasound provoked plasma membrane injury in a low percentage of cells (10% versus 4% in the case of untreated cells) demonstrating that, in our conditions, the necrotic action of ultrasound was very weak. Interestingly, 5 hours after the treatment, 5% of cells were annexin-V positive which reached 11% after 18 hours, demonstrating the ultrasonic induction of apoptosis in K562 cells. We also established differences between the susceptibility of normal and malignant cells to ultrasonic treatment. The results presented in Figure 2B demonstrate that normal

mononuclear cells are scarcely sensitive to ultrasonic treatment under the conditions used in our study. 5h post US treatment, a statistically significant increase in the number of apoptotic cells was observed in the case of K562 cell line ( $2.5 \pm 0.5$  fold increase of the spontaneous apoptosis observed in untreated cells). However, ultrasonic treatment had no effect on normal cell apoptosis ( $1.08 \pm 0.2$  fold increase).

Depletion of cellular glutathione content by ultrasonic treatment:

A flow cytometric method was used to evaluate the cellular content of glutathione (GSH). The nonfluorescent probe 5-chloromethylifluorescein (CMFDA) binds avidly to -SH groups producing brightly fluorescent conjugates. Under the staining conditions used, fluorescence is correlated with the cellular content of GSH. Since dead cells that had lost the capacity to exclude propidium iodide showed low level of CMFDA fluorescence, this population was gated out from further analyses. As shown in figure 3, directly post-US treatment, a subpopulation appeared with lower GSH level than that observed in untreated cells. (more than 50% of cells displaying a low level of GSH). Successive treatments indicate a larger GSH depletion. The results, expressed in % of cells displaying a GSH level comparable to untreated cells, demonstrate clearly that this US treatment is associated with GSH depletion. Moreover, it has been suggested that the reduction of cellular glutathione level seems to precede cell death in which oxidative stress is involved.

Mitochondrial damage:

The early disruption of mitochondrial transmembrane potential ( $\Delta\phi_m$ ), preceding advanced DNA fragmentation has been observed in several models of cell apoptosis (26). Figure 4 shows the changes of US-treated K562 cells stained with DIOC6. Ultrasonic treatment is accompanied by an increase of cell population displaying a low  $\Delta\phi_m$ . Indeed, 2h after the US treatment, a population of cells displaying a

reduced DIOC6 incorporation (respectively 15 and 42% of  $m^{\text{low}}$  cells after one or two US treatments) is evidenced. The drop of mitochondrial potential was very clear 5 hours after the ultrasonic treatment with more than 50% of  $m^{\text{low}}$  in the case of two successive US treatments. 18h after the US treatment this effect was less obvious probably due to the proliferation of non-apoptotic K562 cells.

DNA fragmentation:

Marking with propidium iodide did not allow us to demonstrate any fragmentation of the DNA (subG<sub>0</sub> cells). This is probably due to the low sensitivity of this test and because the effect of the ultrasound was masked by the normal proliferation of healthy cells.

Indeed, 18 h after the US treatment, more than 50% of the cells are in phases S and G<sub>2m</sub>. Nevertheless, cells presenting condensation and fragmentation of DNA were observed after 18 hrs, as evidenced by morphological analysis. It should be noted that the irradiation energy conditions as well as the intracellular topical photosensitiser concentrations were very low compared to classical irradiation, which can only occur in the presence of added photosensitisers. Irradiation times are also very short. In the literature, DNA fragmentation is not a conclusive argument nor is it a necessary and sufficient condition to proof apoptosis.

Effect of successive treatments

After one treatment, the level of apoptotic cells observed is twice that of the control. A necrotic effect of 5 to 10% is observed which is well below that found when using drugs or the PDT treatment. With successive irradiations, under the same conditions (7mW/ml, 20 sec) and at different intervals, apoptosis of K562 cells increases to 20% after two irradiations (Figure 5A and B). Further treatments at intervals of 5, 10 or 60 minutes have identical apoptotic effects, demonstrating that the cumulative effect

depends on the number of treatments and that these can cumulate rapidly without negative side effects. Glutathione depletion and the modification of mitochondrial potential confirm the efficacy of these successive treatments. Morphological variations observed after US treatment are shown in figure 5C. While an important formation of vacuoles , shortly after the treatment and the condensation and fragmentation of the DNA are readily observed 18h after the re-treatment. After one treatment, the morphological effect is less marked probably due to masking by the proliferation of cells unaffected by US.

Ultrasonic Parameters:

• *Frequency ranges*

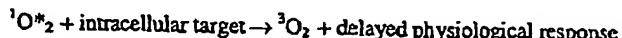
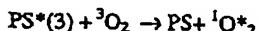
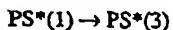
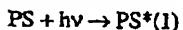
Ultrasonic effects at 2 different frequency ranges have been compared : 35 kHz and 1.8 MHz. At 35 kHz and a higher energy level, phenomena such as those found at 1.8 kHz have not been observed. At 1.8 MHz, to avoid necrosis, the power levels and times of exposure are low (power below 7 mW/ml and exposure time 5 sec for 2.5 ml of solution). At 35 kHz and with power at about 0.22 W/ml, apart from the phenomenon of necrosis, no morphological modification, of the surviving cells is observable. On the contrary, when 1.8 MHz conditions are applied, granular structures and vacuoles can be seen to appear as described above (fig.5C), while there is little necrosis. Furthermore, only the cells treated at high frequency go into apoptosis, the induction of this phenomenon depending on the power and the duration of the radiation (figure 6). It is interesting to note the existence of a power threshold (<5mW/ml) below which the US, in our conditions, have no effect on the cells. At the level of 7 mW/ml, it seems that the effect is optimal.

- *Liquid / air interface*

It has been shown that the effects induced by the ultrasound on leukaemia cells only work in the presence of cavitational nucleators such as albuminous spheres (14). However, even at high frequencies with higher energy and exposure times (3-5 W/cm<sup>2</sup> 30 sec), they only lead to the necrotic lysis of the cells. In our system, with low power, the destruction of cancerous cells is reduced in the absence of bubbles in the ultrasonic field (figure 7). The sensitivity to ultrasound seems also to depend on the type of cell ; K562 cells and the B-CLL cells are the most sensitive compared to HL-60 cells and normal mononuclear blood cells.

## DISCUSSION

The present study demonstrates for the first time the induction of apoptosis in cancer cells by a low energy ultrasonic treatment A mechanism coherent with all our SLDT observations is given in figure 8. It is a type II photochemical reaction :



The sonoluminescence created by the ultrasound activates endogenous photosensitisers which then transfer their energy to fundamental triplet oxygen to form the highly reactive singlet oxygen. When this singlet oxygen reacts, the excess energy is liberated in the form of phosphorescent luminescence . This in turn reinforces the photosensitising effect and creates an autocatalytic cycle of photon production. This mechanism explains the immediacy of the observed SLDT effect and the low levels of the energy initially applied.

In classic ultrasonic irradiation conditions, the direct destructive cavitation effects dominate the sonoluminescence which of course is weak in the absence of an air/liquid interface injected into the medium. The fact that there are no ultrasound effects in our system in the absence of bubbles suggests the major role played by  ${}^1\text{O}_2$  in the SLDT system. Production of  ${}^1\text{O}_2$  as a result of its very short lifetime, is related to the local fundamental oxygen ( ${}^3\text{O}_2$ ) cell concentration. Thus aerobic conditions favours the  ${}^1\text{O}_2$  production within cells. The luminescence wave-lengths specific to singlet oxygen (634 nm, 762 nm and the infrared phosphorescence at 1269 nm) provokes characteristic effects within living cells such as the increase in ATP

synthesis and the perturbation of mitochondrial potential. Evidence against singlet oxygen formation has been presented by Miyoshi et al (27) but the data are only consistent with a long and highly energetic US exposure leading to an accumulation of sensitizer-derived free radicals either by direct pyrolysis or due to reactions with H<sup>•</sup> or OH<sup>•</sup> radicals formed by pyrolysis of the water solvent. Moreover, the radical formation yield, at the frequency used by this author (48 kHz), is known to be much higher than at the 1.8 MHz frequency used for our SLDT experiment. New singlet oxygen specific detection techniques are now available (28,29) and could be used to ascertain the exact mechanism involved but, due to the SLDT extremely low energies and exposure times, their physical detection level will be questionable. The major targets of PDT are cell membranes and it is the same for SLDT, except that SLDT is the only one to act directly within the interior of the cells. The photodynamic damage induced before the exposure to ultrasound has the effect of increasing cellular lysis without affecting the clonogenicity of the surviving cells ; whereas photo-induced damage following an exposure to ultrasound lowers the viability of those cells that have survived the ultrasonic treatment (30). Sonoluminescence being in our case the inducing physical phenomenon, it is normal that our results can be compared to photolytical damages suffered prior to an exposure to classic ultrasound. With this SLDT technique, according to the doses and conditions of ultrasonic irradiation, one can either lyse the cells or induce apoptosis without any delayed effect on the viability of the surviving cells .

All these elements combine to relate the effects obtained to those of the PDT technique rather than those found with SDT, but this is achieved without the necessity of classical photosensitizing drugs. Within the cells it is the endogenous photoabsorbing molecules such as the porphyrins and the flavoproteins who play the

photosensitizing role (31). Cell irradiation at certain wavelengths can activate some of these endogenous molecules which then take part in a metabolic reaction not directly related to the luminescence (15). The physiological effects obtained with techniques such as phototherapy depend at the same time on the radiation dose, on the nature of the photosensitizers used, on their concentration and on their localisation. With PDT, oxidative damage to DNA is related to concentrations of porphyrins in the cells, apoptosis being only observed for weak concentrations (32). The relative concentrations in external PS are fairly weak in the traditional therapies but on the other hand, preferential sites exist in the cells characterised by important local concentrations of PS. In our technique, the net effects of the ultrasonic action suggest that endogenous PS must be implicated in the structures where their local concentration is high (Table 1). Different mechanisms and results are involved according to whether the PS are injected into the cells themselves or if they are located in the extracellular medium (33). In fact, the membranes stop the diffusion of extracellular PS and the mechanism is then confined to the cell periphery. However, any endogenous PS will be immediately involved in the intracellular mechanisms. The cell bound PS are a much more effective in photodynamic activation than the free extracellular PS , and it has been observed that the lipophilic PS are the most effective (34). According to their nature, the PS are located differently within the cell, for example, Tolyporphine, one of the most effective PS found to date in PDT, is localised almost exclusively in the perinuclear region, specifically in the endoplasmic reticulum. Other types are to be found in the lysosomes or in the mitochondria (16,35). These endogenous PS are localised mainly in the membrane structures such as lysosomes, mitochondria, nuclear membranes and the microsomes of the endoplasmic reticulum of which the relative surface represents nearly 50 % of the

membrane surface of the cells. Under the soft SLDT conditions to which they are subjected, the healthy cells are much less sensitive to ultrasound than the cancerous cells. However, at higher powers and at those frequencies, all the cells are lysed. Even though this difference in behaviour between the healthy and cancerous cells cannot be related to a difference in the localisation of the endogenous PS but probably due to a modification of the fundamental cell mechanisms. For SLDT, a similar sequence of events to that induced by PDT has been observed ; a rapid induction provoked by the formation of  $^1\text{O}_2$  having on the one hand an effect on the mitochondrial membranes (drop of mitochondrial potential) and, on the other hand, provoking a lipidic oxidation of the membrane, as shown by an evaluation of the cellular GSH level. Specific tests for the apoptosis detection (annexin-V/PI assay) show the very rapid mechanism of SLDT and show this to be a specific mechanism that substitutes for necrosis.

DNA fragmentation was not evidenced, even though morphologically, cells that presented a condensation and fragmentation of DNA were observed after successive treatments. However in the literature, DNA splitting does not seem to be a necessary and sufficient condition to prove apoptosis. Recently, another ultrasonic technique has been described (5). This therapeutic ultrasound (750kHz) is characterised by a high intensity delivery to generate cavitation. Besides a large amount of necrosis, apoptosis is induced in the surviving cells. It seems that the mechanisms, similar to those produced by  $\gamma$ -irradiation, are somewhat different: inhibition of the cell proliferation , no G2 phase induction.

Nor has Caspase-3 activation been demonstrated during SLDT treatment although this seems essential for apoptosis induction by  $^1\text{O}_2$  (36). In contrast to PDT, in our system, the  $^1\text{O}_2$  does not appear to activate apoptosis pathway via the caspases. This suggests

that either the involved sites are of a different nature or quite simply that other routes are preponderant and more immediate. Recently, other mechanisms of cell death without activation of the caspases nor nuclear signs have been described (37).

Also, it must be noted that, in contrast to PDT, the aggression originates from within the cell and not from the external environment. The SLDT mechanism implies the formation of species that are difficult to detect directly because of their extremely weak concentration, and their very short lifetime. These species being effective at minutely small doses place us in a similar domain to the prostaglandins or others (38).

The effectiveness of the system lies in the fact that it bypasses the primary intercellular signals and that one enters the process more rapidly and at a more advanced stage without having to wait for signal induction by messenger molecules. The low power conditions allow the amount of the necrosis to be reduced while specifically favouring apoptosis.

The supernatant of the cells submitted to one or several US treatments are unable to induce apoptosis in healthy K562 cells, thus demonstrating that the effects generated do not originate from chemical messengers secreted by necrotic cells.

At the chosen frequencies, the US do not directly generate free radicals as could those originating in the sonolysis of the solvent or those from the addition of molecules such as DMSO, DMF which generate a sonodynamic action on leukaemia cells (39,40). The cancer therapy presented here offers a mechanism for the smooth elimination of sick cells by means of apoptosis which is preferred for non-inflammatory reasons.

The extension of the technique can be envisaged to the problems of extra-corporeal circulation in the treatment of different types of leukaemia, without use of chemotherapy. Its use can also be foreseen for treatment of pathologies that involves

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oxidative stress or singlet oxygen, without the need to add neither reactive agents nor photosensitisers.

**LEGENDS**

**Figure 1 :** Description of the apparatus used for cell irradiation throughout this study.

**Figure 2 :** (A) Flow cytometric analysis of apoptosis in K562 cells. The cytograms represent the evolution of green (Annexin-V) and red (PI) fluorescences for various times post-US treatment.

(B) Comparison between the US induction of apoptosis in normal peripheral blood mononuclear cells and malignant K562 cells. Each bar represents the mean of three independent experiments. Phosphatidylserine exposure was measured at 5h and 18h post US-treatment by binding of annexin V and the results are expressed as fold increase of apoptosis in comparison with the spontaneous apoptosis of untreated cells.

**Figure 3 :** Effect of ultrasonic treatment on cellular glutathione levels. Intracellular GSH content was evaluated by the method described under materials and methods. Dead cells that had lost the capacity to exclude propidium iodide were gated out from glutathione analysis. Data given are expressed in percentage of cells displaying glutathione level comparable to untreated cells. The example is representative of 3 independent experiments.

**Figure 4 :** The DIOC6 staining of K562 cells treated or not by ultrasound. The histograms represent cell number (counts) versus green fluorescence intensity (FL-1): cells were incubated with DIOC6 followed by immediate cytofluorometric analysis.

Figure 5 : Effect of successive US treatments on the viability of K562 cells.

- (A) Changes of phosphatidylserine distribution according to time and successive US treatments. Results are expressed as percentage of cells stained with annexin-V-FITC (PI negative cells). PT = post-treatment.
- (B) Detection of apoptosis by annexin-V/PI assay in K562 cells treated by successive irradiations.
- (C) Morphological features of May-Grünwald Giemsa-stained K562 treated or not by US. Arrows indicate cells with morphological changes such as vacuolisation and pyknosis.
  - Untreated cells : (a) (X 500).
  - 1 US treatment : (b) post (X 500), (c) 5h (X 500).
  - 2 US treatments : (d) post (X 1000), (e) 5h (X 1000), (f) 18h (X 500).

Figure 6 : Induction of apoptosis in K562 cells by ultrasonic treatment. Influence of power and time of treatment. Apoptotic cells were evaluated by annexin-V/PI assay as previously described.

Figure 7 : Effect of ultrasound on the viability of different cell types in the presence or absence of air bubbling. Cells were treated during 15 sec at a power of 25 mW/ml. The viability of cells, post-treatment, was evaluated by the trypan blue exclusion test.

Figure 8 : Proposed mechanism for the sonoluminescence dynamic therapy (SLDT).

The sonoluminescence created by the ultrasound activates cellular endogenic photosensitisers which then transfer their energy to fundamental triplet oxygen to form the highly reactive singlet oxygen. When this singlet oxygen reacts, the excess energy is liberated in the form of a phosphorescent luminescence reinforcing the photosensitising effect (autocatalytic cycle).

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Table 1

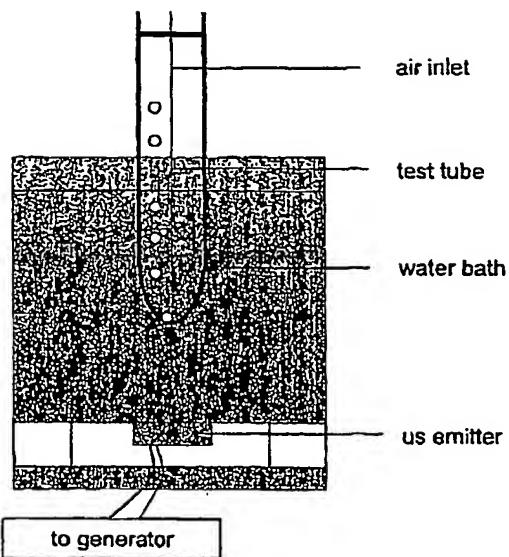
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	Localization	Relative concentration	
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SDT	Extracellular	LOW	NECROSIS > APOPTOSIS
SDT	Systemic Intracellular	LOW	NO EFFECT
SLDT	Topic Intracellular	HIGH	APOPTOSIS > NECROSIS

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Fig 1



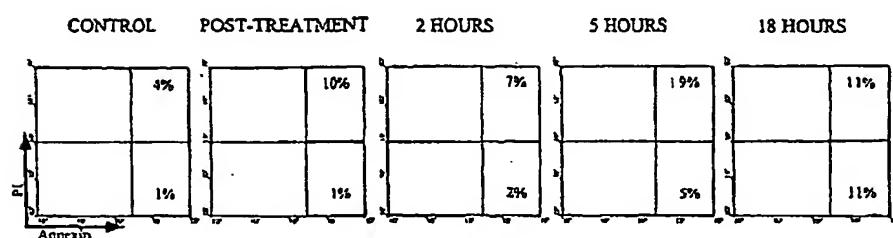
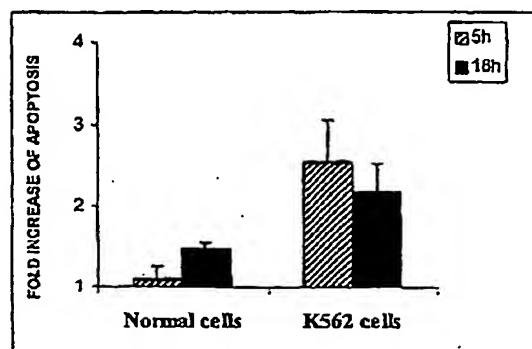
**Fig 2A****Fig 2B**

Fig 3

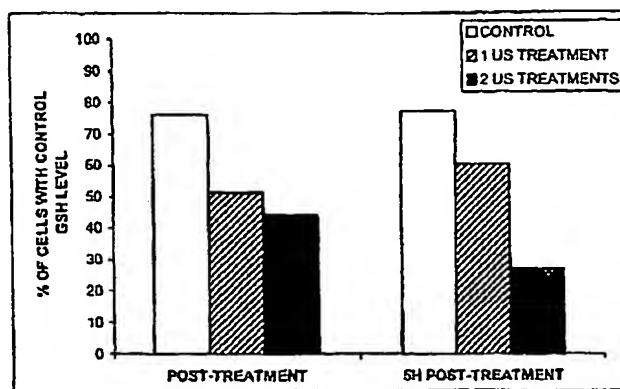
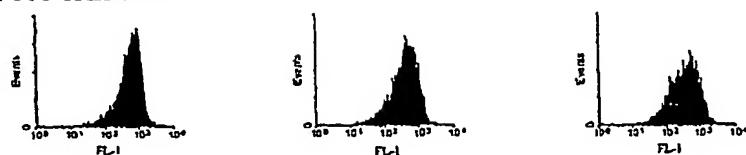
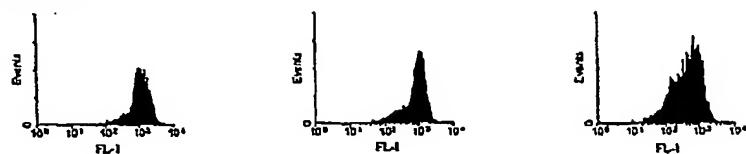


Fig 4

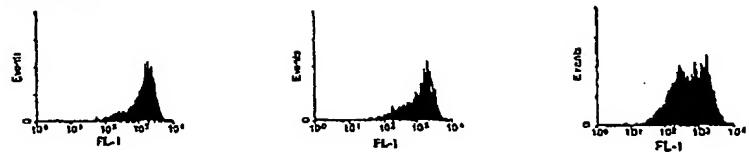
## POST-TREATMENT



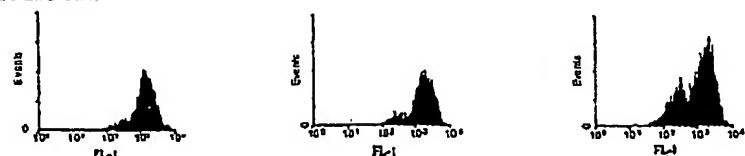
## 2 HOURS



## 5 HOURS



## 18 HOURS



Untreated cells

Treated cells  
(1 treatment)Treated cells  
(2 treatments)

Fig 5

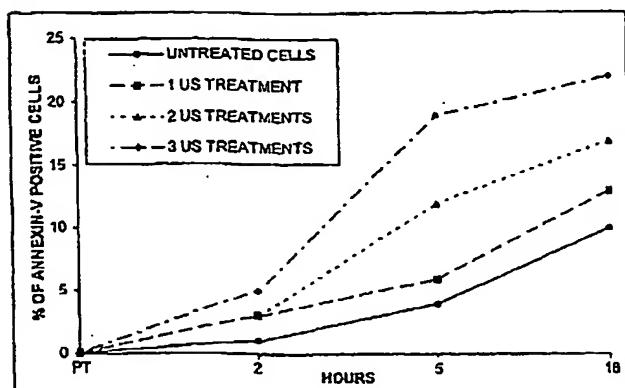
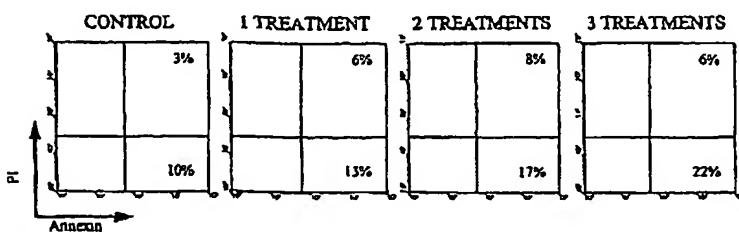
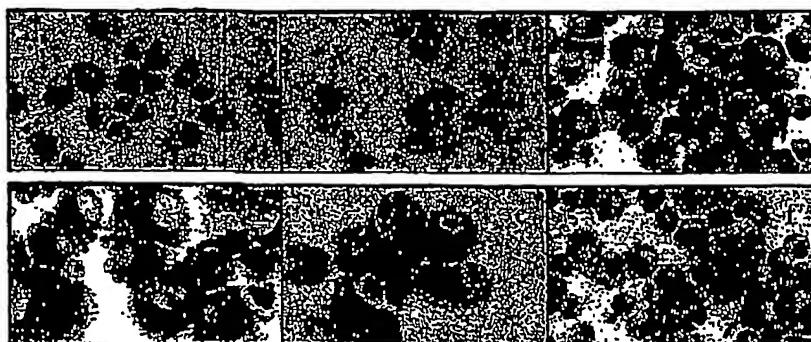
**A****B****C**

Fig 6

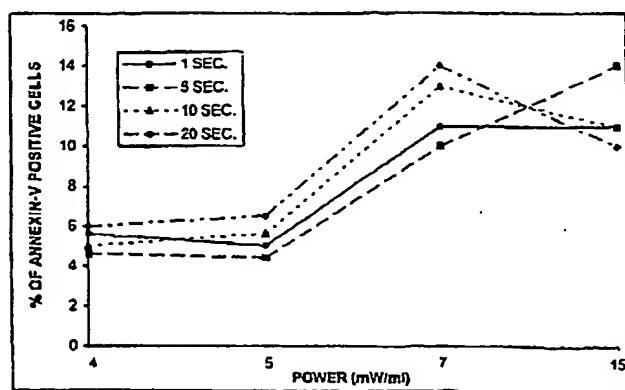
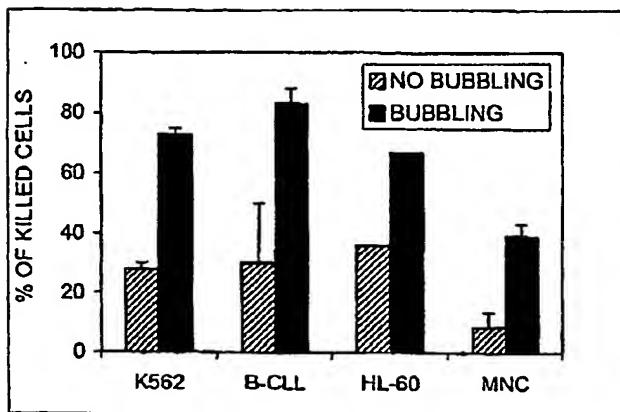


Fig 7



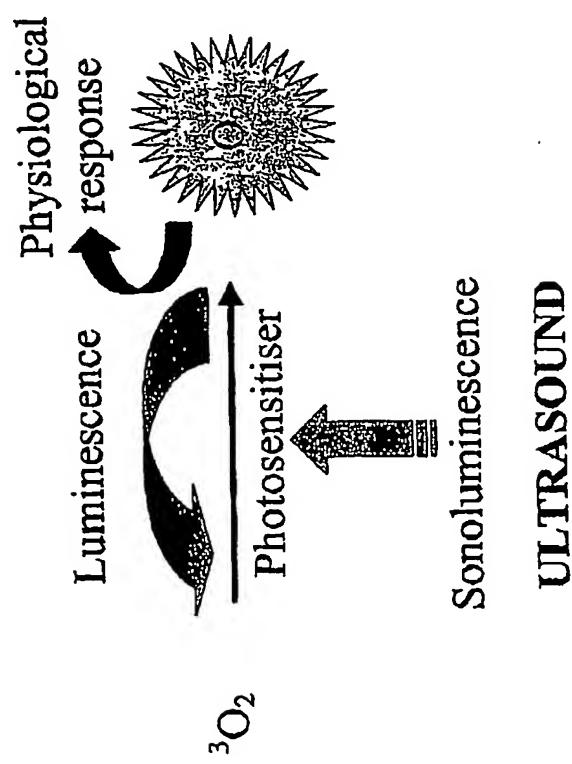


Fig 8

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